Application of autologous periosteal cells for the regeneration of class III furcation defects in Beagle dogs

Jun Jiang · Xiaohong Wu · Minkui Lin ·
Nghiem Doan · Yin Xiao · Fuhua Yan

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Abstract The aim of this study was to evaluate the healing of class III furcation defects following transplantation of autogenous periosteal cells combined with β -tricalcium phosphate (β -TCP). Periosteal cells obtained from Beagle dogs' periosteum explant cultures, were inoculated onto the surface of β -TCP. Class III furcation defects were created in the mandibular premolars. Three experimental groups were used to test the defects' healing: group A, β -TCP seeded with periosteal cells were transplanted into the defects; group B, β -TCP alone was used for defect filling; and group C, the defect was without filling materials. Twelve weeks post surgery, the tissue samples were collected for histology, immunohistology and X-ray examination. It was found that both the length of newly formed periodontal ligament and the area of newly formed alveolar bone in group A, were significantly increased compared with both group B and C. Furthermore, both the proportion of newly formed periodontal ligament and newly formed alveolar bone in group A were much higher

than those of group B and C. The quantity of cementum and its percentage in the defects (group A) were also significantly higher than those of group C. These results indicate that autogenous periosteal cells combined with β -TCP application can improve periodontal tissue regeneration in class III furcation defects.

Keywords Autologous periosteal cells · Alkaline phosphatase · Class III furcation defects · Periodontal regeneration · Tissue engineering · β -Tricalcium phosphate

Abbreviations

MSC	Mesenchymal stem cells
DMEM	Dubbleco's-modified Eagle's memoleculum
SEM	Scanning electron microscope
β -TCP	β -Tricalcium phosphate
e-PTFE	e-Polytetrafluoroethylene

J. Jiang · X. Wu · M. Lin · F. Yan (✉)
School and Hospital of Stomatology, Fujian Medical
University, 246 Yangqiao Zhong Road, 350002 Fuzhou,
Fujian, People's Republic of China
e-mail: fhyan2005@126.com

N. Doan · Y. Xiao
Institute of Health and Biomedical Innovation,
Queensland University of Technology, Brisbane,
QLD, Australia

Introduction

Periodontitis is considered as subgingival inflammation caused by bacterial infection Shimizu et al. (2009). It affects the periodontal supporting tissues including periodontal ligament, cementum, and alveolar bone. Periodontitis affects the junction of multi-rooted tooth, initially with tissue destructions then

gradually with further bone loss and eventually furcation involvement will be occurred. Aukhil (1991) pointed out that periodontal regeneration aims at restitution of supporting periodontal tissues loss due to periodontal diseases. However, furcation involvement is difficult to treat properly by conventional periodontal therapy or surgery. Jepsen et al. (2002) and Needleman et al. (2002) found that guided tissue regeneration (GTR) could promote periodontal regeneration and new attachment formation, especially in class II furcation defects and bone base pockets. But its therapeutic effect on class III furcation defects was limited. Pontoriero et al. (1992) reported that GTR was capable of closing class III furcation defects successfully in a dog model. However, the regeneration of class III furcation defects was always incomplete. It is suggested that this treatment is not effective for large class III furcation defects. De Bari et al. (2006) found that periosteal cells were clonogenic, displayed long telomeres and expressed markers of MSCs, regardless of donor age. Under specific conditions, both parental and single-cell-derived clonal cell populations could be differentiated to chondrocyte, osteoblast, adipocyte, and skeletal myocyte lineages in vitro and in vivo. Groeneveld et al. (1994) have also approved that periosteum cells could form cartilage or bone in vivo. When periosteum was cultured in direct contact with bovine dentin slices in the presence of 10 mmol L^{-1} β -glycerophosphate, a fibrillar acellular cementum could be formed in these slices after 2 weeks. Steiner et al. (2007) indicated that periosteum was able to form alveolar bone, cementum and periodontal ligament when it was transplanted into periodontal defects. Mizuno et al. (2006) repaired Class III furcation defects in Beagle dogs by grafting autologous periosteal cells which was cultured from membrane derived from periosteum. They found that these membranoid promoted regeneration of periodontal tissue, and was able to form bone and worked as a barrier membrane in the regeneration of periodontal tissue. In the study conducted by Matsumoto et al. (2009), where β -TCP was used as scaffold material, they have proven that these membranoid are biodegradable and osteoconductive. To investigate the potential application of periosteal cells in periodontal regeneration, Beagle dogs were served as models for class III furcation defects in this study. Autogenous periosteal cells were harvested from the

same dog, and then combined with β -TCP for the transplantation into class III furcation defects of the same Beagle dog. After 12 weeks, periodontal tissue regeneration was assessed by histology and histomorphometry.

Materials and methods

Preparation of periosteum cells and β -TCP composite

Four healthy Beagle dogs (Sichuan Academy of Medical Sciences Sichuan People's Hospital Experimental Animal Research Institute) weighted between 9.5 and 10.5 kg were used for this study. Small pieces of periosteum around $5 \text{ mm} \times 4 \text{ mm}$ in size, were harvested from the mandible of each dog during the time of establishing the furcation defects (detailed in the "Surgical procedure"). These periosteal explants were then cut into smaller pieces of about 1 mm^2 , and placed into the bottom of 6-well plates which were pre-wetted with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and penicillin–streptomycin (100 IU mL^{-1}). The samples were then covered with $25 \text{ mm} \times 20 \text{ mm}$ cover glass. The periosteum was cultured in a humidified atmosphere of 5% CO_2 at 37°C . The medium was changed every 3 days until the outgrown cells were subconfluent, after that non-adherent cells were washed off with PBS and the adherent cells detached with trypsin–EDTA (Gibco, USA) and sub-cultured in a 10-cm dish.

β -tricalcium phosphate (β -TCP) (Shanghai Bio-lu Biomaterials Company Limited, China) was used as scaffold material in this study. β -TCP is composed of CaO and P_2O_5 at the ratio of 1.5 for Ca/P , similar to the inorganic composition of bone. The β -TCP used in this study was granular porous ceramic and their diameters were less than 1 mm. Prior to in vivo transplantation, the β -TCP was sterilized with ethylene oxide gas, soaked in DMEM in 6-well plates (0.6 g per well, overspread in the well with 1 mm in thickness) for 24 h to facilitate cell-scaffold incubation. The third passaged periosteal cells were incubated with β -TCP at $5 \times 10^6 \text{ cells mL}^{-1}$ in 6-well plates in 1 mL culture media per well. The cell and β -TCP complex was cultured in DMEM, supplemented

with 10% FBS, and cultured at 37 °C with 5% CO₂ for 48 h.

Surgical procedure

Six weeks prior to the cell transplantation, class III furcation defects were created surgically at the second, third and fourth mandibular premolars of Beagle dogs by using slow-rotation diamond burs. The alveolar bone from each premolar (₂P₂, ₃P₃, ₄P₄) was removed, creating a “horizontal” pattern of bone loss. According to the protocols by Yan et al. (2003) and Fernandes et al. (2005), the furcation defects were approximately 3 mm wide and 4 mm high and were filled with gutta-percha to promote inflammatory reaction. Granulation tissue was removed and the root surface was thoroughly debrided by curets. Notches were placed on either side of the root at the top of the bone defects. Defects were then covered with e-polytetrafluoroethylene (e-PTFE) membrane completely. The e-PTFE membranes were fixed with surgical gel (Suncon, China). Mucoperiosteal flaps were subsequently repositioned and sutured. All dogs were fed on soft food to prevent traumas of mastication to the surgical areas. Six weeks later, the animal model of class III furcation defects were confirmed (Fig. 1).

All teeth were randomly divided into three groups. Each furcally involved premolar was then assigned to one of three treatment groups: group A (e-PTFE plus β -TCP and periosteal cells), group B (e-PTFE plus β -TCP), group C (e-PTFE). Mucoperiosteal flaps were

raised and sutured with coronally reposition flap. No oral hygiene was instituted during the treatment. Six weeks later, the e-PTFE membranes were removed and assessed by scanning electron microscope with JEOL JSM-6330F field emission (Philips XL30, NLD).

Histological processing

The animals were euthanized 12 weeks after surgery. The jaw of each animal was removed and specimens containing the experimental areas were harvested and placed in 20% buffered formalin. The radiolucency and density of the experimental defects in alveolar bone were assessed by X-rays. Specimens were then decalcified with 10% ethylenediaminetetra acetic acid (EDTA Gibco USA) for 2 months and then dehydrated in ethanol, embedded in paraffin, and serially sectioned (to 4 μ m thickness) in the mesio-distal direction. Five sections collected from the central area of each furcation were selected from for further analyses. All sections were stained with hematoxylin-eosin and Mallory trichrome dye. The histological specimens were analyzed by a light microscope fitted with a digital camera. The linear distance and area, were measured by an analytic software (Olysia Bioreport3.2; Olympus, Japan). The measurements include: (1) TDL (total defect length): the distance between the notches on the mesial and distal roots. (2) NC (new cementum formation): the distance of root surface which was covered by newly formed cementum on the mesial and distal roots. (3)

Fig. 1 Surgical procedure in dogs. Surgically induced class III furcation defect at the second, third and fourth mandibular premolars. The furcation defects were approximately 3 mm

wide and 4 mm high and filled with gutta-percha to promote inflammatory reaction (*asterisk*)

Fig. 2 Schematic diagram for histological processing. *N*: notches on root surface; *A, B*: top of newly formed cementum; *C, D*: top of periodontal ligament; *E*: top of root furcation; *NBA*: new bone area; *STA*: soft tissue area, the area filled with connective tissue and/or epithelial tissue; *NFA*: non-filled area. Calculation method for each index: TDL(total defect length) = the sum of N1E and N2E; TDA(total defect area) = the sum of NBA, STA and NFA; NC(new cementum formation) = the sum of N1A and N2B; NP(new periodontal formation) = the sum of N1C and N2D; $NC\% = NC/TDL \times 100\%$; $NP\% = NP/TDL \times 100\%$; $NBA\% = NBA/TDA \times 100\%$; $STA\% = STA/TDA \times 100\%$; $NFA\% = NFA/TDA \times 100\%$

NP (new periodontal formation): the distance of root surface that periodontal ligament fibers had penetrated in between the notches between the mesial and distal roots. (4) TDA (total defect area): total defect area between the notches on the mesial and distal roots. (5) NBA (new bone area): the area filled with mineralized tissue. (6) STA (soft tissue area): the area filled with connective tissue and/or epithelial tissue. (7) NFA (non-filled area): the area filled with nothing or occupied by dental plaque in the furcation defects. All distances were measured in millimeters (mm), and areas were measured in square millimeter (mm²). (Fig. 2).

Statistical analysis

Data obtained from linear measurements, area measurements and the percentage of regenerated tissue in the original defects, were expressed as means and standard deviation. One-way analysis of variance (ANOVA) was used for statistical evaluation. Student–Newman–Keuls (SNK) test was used to compare differences between groups. *p*-values less than 0.05 were considered statistically significant. These

analyses were performed using Statistical Package for Social Science 10.5 for windows (SPSS 10.5, USA).

Results

Clinical observations

Visual inspection: Upon visual inspection, parts of the barrier membranes appeared to separate from tooth cervix in all experimental animals, the coronal areas were not covered completely with gingiva while the unexposed membranes adhered tightly to tooth cervix. Closer observation of the gingiva adjacent to the membrane revealed that the gingiva attached and grew well into the central portion of the membranes. Once the e-PTFE membranes were removed, it became apparent that newly formed granulation tissues was detected in the defects. **SEM inspection:** At the coronal portion of the tested site, evidence of orderly arranged collagen bundles, and bacterial colonies were found on the membranes (Fig. 3a). In the middle portion of the defects, it was obvious that a large quantity of fibre bundles adhered on the surface of the membranes in conjunction with well distributed and scattered erythrocytes were observed (Fig. 3b). In the lowest portion of the defect, the microfibrils on the membrane appeared to join to each other and there were clear evidence of porous structures between the microfibrils, as well as a few inflammatory cells. (Fig. 3c).

Efficacy of treatment

All animals survived from surgery without any adverse events. However, furcal perforation happened to a few teeth during preparation of defect model as well as during treatment (one in group A and one in group C). Furcation over preparation occurred in a number of teeth (one in group A, two in group B, and one in group C). The furcation defects, which were over prepared and fail to meet the inclusion criteria in this study, were excluded. For all teeth included in this study, their furcation defect appeared to heal reasonably well with healthy gingival attachment. However, in a number of teeth, a small amount of calculus and plaque were detected in conjunction with minimal gingival recession (<2 mm).

Fig. 3 SEM inspection of ePTFE membranes. **a** In the coronal portion, collagen bundle and bacterial plaques appeared on the surface (arrowheads) ($\times 5,000$); **b** In the middle portion, erythrocyte (arrowheads) and fibre bundles could be seen

($\times 1,000$); **c** In the bottom portion, membrane structure of the micropore could be seen, and a few collagen fiber appeared on the surface (arrowheads) ($\times 1,000$)

Radiographical analyses

Dental radiographs were employed to assess mandibular bone defects 12 weeks after implantation. These radiographs showed evidences of bone regeneration as well as resorption. Areas of radiolucencies were clearly visible at bone-defect sites in group B and C. In group A, the largest amount of bone regeneration was detected by X-rays at the bone-defect sites (Fig. 4).

Histological analyses

Histological observation of the defect sites showed that the epithelium was found to surround all

regenerating tissue, and the newly formed connective tissue was detected in the root furcation. Greater amount of newly-formed alveolar bone was observed in group A than in groups B and C. Group C exhibited moderate or severe soft tissue inflammation. The newly formed cementum containing cementoblasts, distributed non-uniformly in all groups. These, in turn, helped uneven and collagen fibers insert into the newly formed cementum. In addition, immature newly formed bone including osteoblasts was observed in all three groups. The newly formed reverse lines showed that there were obvious boundaries between the newly formed bone and the existing bone (Fig. 5).

Fig. 4 Radiographs of the mandibular bone defects 12 weeks after implantation. **a** In group A, the largest amount of bone was formed at the bone-defect sites. **b** and **c** Radioparent

regions were clearly visible at bone-defect sites in group B and C, defects were not filled completely

Fig. 5 Histological analyses of three groups. **a, d, g** In group A, the defects were mainly occupied by newly formed bone and connective tissue, with a few epithelial tissue; **b, e, h** In group B, the defects were filled with connective tissue, epithelial tissue and newly formed bone which was less than in group A; **c, f, i** In group C, the defects were mainly occupied by connective tissue, and newly formed bone was observed on the notch of root surface. New bone which contained osteoblasts, cell-like newly formed cementum (NC) (yellow arrowheads in **d, e, f**) and NP perforant path (black arrowheads in **g, h, i**) could be observed in the three groups. (NBA = new bone area, NB = new bone formation, NC = new cementum formation, NP = new periodontal ligament formation, STA = soft tissue area, ETA = epithelium tissue area) (**a, b, c**, HE staining: $\times 40$; **d, e, f**, HE staining: $\times 200$; **g, h, i**, Mallory trichrome staining: $\times 400$)

Results of linear measurements

The one-way analysis of variance and multiple comparison test indicated that the TDL among these three groups had no significant difference ($p > 0.05$). The length of NC and NP had no significant difference between groups A and B, neither between groups B and C ($p > 0.05$). But the length of NC and

NP in group A were significantly higher than in group C ($p < 0.05$) (Table 1).

Results of area measurements

The analysis of variance and multiple comparison test showed that there was no significant difference for the TDA and STA among these groups ($p > 0.05$).

Table 1 Results of linear measurements after surgery

Group	A	B	C	<i>p</i>
TDL (mm)	10.81 ± 1.04	10.21 ± 1.33	10.83 ± 1.67	<i>p</i> > 0.05
NC (mm)	5.53 ± 1.34*	4.71 ± 1.47	3.37 ± 1.21	<i>p</i> < 0.05
NP (mm)	3.71 ± 1.82*	2.34 ± 0.47	1.33 ± 0.31	<i>p</i> < 0.05

* Compared with group C, *p* < 0.05

TDL, NB, NC and NP of each groups (*n* = 6)

Table 2 Results of area measurements after surgery

Group	A	B	C	<i>p</i>
TDA (mm ²)	10.38 ± 1.79	10.11 ± 2.05	10.59 ± 3.52	<i>p</i> > 0.05
NBA (mm ²)	6.21 ± 2.18* [#]	3.71 ± 2.00	2.35 ± 1.26	<i>p</i> < 0.05
STA (mm ²)	3.37 ± 2.35	4.85 ± 1.28	5.41 ± 4.54	<i>p</i> > 0.05
NFA (mm ²)	0.77 ± 0.47*	1.72 ± 0.74*	2.78 ± 1.22	<i>p</i> < 0.05

* Compared with group C, *p* < 0.05; [#] Compared with group B, *p* < 0.05

TDA, NBA, STA and NFA of each group (*n* = 6)

The NBA in group A was significantly higher than that of group B and C (*p* < 0.05), the NFA in group B was significantly lower than that of group C (*p* < 0.05) (Table 2).

The percentage of regenerated tissue in the original defect

The percentages of the length or area of all newly formed tissues in the original defect were calculated. The analysis of variance and multiple comparison test showed that NP and NBA% in group A were significantly higher than that in groups B and C, respectively (*p* < 0.05); NC% in group A was

significantly higher than that of group C; STA% in group A was lower than that of groups B and C (*p* < 0.05); NFA% in group A was significantly lower than that of group C (*p* < 0.05). NBA% in group B was significantly higher than that of group C (*p* < 0.05), but STA and NFA% in group B were significantly lower than that of group C, respectively (*p* < 0.05) (Table 3).

Discussion

Hutmacher and Sittinger (2003) pointed out that periosteal cells could differentiate to bone when implanted in vivo, and had the potential for tissue engineering in bone reconstruction. Furthermore, De Bari et al. (2006) demonstrated that, periosteal cells could also differentiate into chondrocyte, adipocyte, skeletal muscle cells and seem to be a favorable kind of cells for periodontal tissue engineering. In this study, the periosteal cell-induced periodontal regeneration were evaluated for the feasibility of autologous periosteal cells transplantation, in repairing class III furcation defects.

In all six experimental teeth of each treatment, it was noted that no significant difference was found in average defect length and area of furcation defects in each group (*p* > 0.05). Lindhe et al. (1995) demonstrated that the periodontal defects were difficult to

Table 3 Percentage of newly-formed periodontal tissue took in the original defect

Group	A	B	C
NC (%)	50.77 ± 8.19*	43.02 ± 8.08	31.67 ± 12.83
NP (%)	33.64 ± 13.83* [#]	22.18 ± 5.19	17.70 ± 5.35
NBA (%)	60.23 ± 18.16* [#]	34.77 ± 15.38*	22.62 ± 7.33
STA (%)	32.59 ± 17.33* [#]	50.11 ± 13.57*	51.81 ± 5.48
NFA (%)	7.19 ± 3.56*	14.68 ± 7.16*	24.61 ± 1.13

* Compared with group C, *p* < 0.05; [#] Compared with group B, *p* < 0.05

Percentage of the length of NC and NP in TDL and the mean percentages of NBA, STA and NFA in TDA (*n* = 6)

repair completely if they were larger than 4 mm. In this study, the heights of defects were more than 4 mm, and the average area in the three groups was 10.62 mm². Pontoriero et al. (1992) pointed out that, in the “horizontal” defects only the bone wall in the bottom supported cells to induce periodontal regeneration. Hovey et al. (2006) demonstrated that plaque was detrimental to traditional surgery and periodontal regeneration. In this study certain amount of plaque accumulation in the surgical sites was found due to the difficulties to keep oral hygiene for the animals, which may be responsible for the interruption of periodontal tissue regeneration and induce incomplete periodontal defect healing.

Regazzini et al. (2004) pointed out that, in GTR, the barrier membrane preserves a space for coronal migration of periodontal ligament cells and endosteal cells from the defect base. Tamai et al. (2007) reported that the β -TCP was widely used as a filling material in bone and cartilage repair. In this study, β -TCP with certain plasticity could prevent subsidence of e-PTFE membrane when it was combined with blood and culture medium, which preserves a space for proliferation of cells. However, β -TCP was displaced in some teeth in this study and the sealability of e-PTFE membrane was compromised, which allowed epithelial cells and connective tissue to grow into the defects. In each group it was found that epithelial cells and connective tissue were detectable in all defects.

In the newly regenerated periodontal tissues, there were evidences of newly formed alveolar bones, cementum and periodontal membrane. This indicated that the most likely pathway for periodontal recovering was to regenerate the functioning periodontium. Our results showed that the regeneration effect of periosteal cells in combination with β -TCP was better than the application of β -TCP alone. Compared with the defect without β -TCP filling, periodontal regeneration was detectable in β -TCP treatment group. This implies the ability to repair class III furcation defects by simple e-PTFE membrane is limited. The implantation of β -TCP served as space maker for the proliferation and differentiation of precursor cells. Furthermore, the osteoconductive nature of β -TCP facilitates bony tissue and blood vessels to grow into the implanted material efficiently. This subsequently promoted bone regeneration as well as restored functions of the defected teeth. Declercq et al.

(2005) have shown that periosteum cells could differentiate into bone and play an important role in bone regeneration and the healing of bony defect or fracture. The same authors also indicated that periosteal cells express markers of mesenchymal stem cells and under specific conditions, they can differentiate to the chondrocyte, osteoblast, adipocyte, and skeletal myocyte lineages in vitro and in vivo. De Bari et al. (2006) and Steiner et al. (2007) have demonstrated that the periosteum cells can take part in the regeneration of cementum, periodontal ligament and alveolar bone. Mizuno et al. (2006) indicated that when the membranoid substance combined periosteum block and periosteal cells, then transplanted into class III furcation defects, could accelerate the regeneration of periodontal tissue. When combined with GTR membrane, Steiner et al. (2007) found that periosteum transplanted to furcation defects could help the regeneration of alveolar bone, cementum and periodontal ligament. Similar results were reported in the application of the periodontal ligament fibroblasts and BMSCs by Yan et al. (2005), Hovey et al. (2006), Gay et al. (2007) and Li et al. (2009). Although the multiple differentiation potential of periosteum cells has been shown, no study has confirmed that periosteum cells can differentiate to cementoblasts. The newly formed periodontal ligament and cementum might be differentiated from transplanted periosteal cells or derived from intrinsic periodontal ligament fibroblasts in the defects. Clearly, this study has demonstrated that the implantation of periosteum cells into the defects can significantly increase the quantity and biological activity of the tissue healing process in the type III furcation model.

Conclusion

Periosteal cells transplanted into the class III furcation defects in dog model showed an accelerated regeneration process of periodontal tissues. However, due to the challenge of cell delivery in the contaminated oral environment, ideal regeneration techniques need to be further developed and transplanted cell differentiation in periodontal defects also requires further investigation.

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